

Engineering Large Animal Species to Model Human Diseases

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Animal models are an important resource for studying human diseases. Genetically engineered mice are the most commonly used species and have made significant contributions to our understanding of basic biology, disease mechanisms, and drug development. However, they often fail to recreate important aspects of human diseases and thus can have limited utility as translational research tools. Developing disease models in species more similar to humans may provide a better setting in which to study disease pathogenesis and test new treatments. This unit provides an overview of the history of genetically engineered large animals and the techniques that have made their development possible. Factors to consider when planning a large animal model, including choice of species, type of modification and methodology, characterization, production methods, and regulatory compliance, are also covered. © 2016 by John Wiley & Sons, Inc.

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INTRODUCTION

The first genetically engineered (GE) large animal species were developed over 30 years ago; however, it is only recently, with the convergence of several key technologies, that large animals are beginning to gain popularity and acceptance. To date, most GE large animal work has occurred in livestock species, with pigs being the most common. Besides the obvious agricultural applications, livestock species are also being pursued for biopharmaceutical production, organs for xenotransplantation, and modeling human disease. It is the latter that will be the general focus of this unit; however, most of the techniques and considerations discussed are relevant across applications.

GE mice have been mainstays of biomedical research since the 1980s. The ability to study the function of a gene in a mammalian setting has made significant contributions to our understanding of basic biology, disease mechanisms, and drug development. Their rel-

ative low cost and ease-of-use make GE mice an attractive choice for these applications. However, mice can also pose significant disadvantages. For example, GE mouse models often fail to recreate important aspects of the human diseases they are modeling, limiting their translational research utility. Conversely, large animal species have long been used as surrogates for humans in biomedical research due to similar physiology, anatomy, metabolism, genetics, and size. With advances in genome engineering and reproductive biology, the desire to utilize improved models of human disease has led to significant interest in developing GE large animal species.

This unit provides an overview of the history of GE large animals and the ever-evolving techniques that have made their development possible. Where appropriate, the major differences between techniques used to develop large animals versus those used in mice are highlighted. Also discussed are issues to consider when developing a GE large animal, such



as choice of species, type of modification, methodology, and characterization activities. An example of a successful GE large animal model is examined, demonstrating the potential impact of a more accurate model. Finally, some of the challenges that still remain for the field of GE large animals are discussed. It is important to note that this overview is not intended to be an exhaustive list of every technique or a how-to protocol. Readers are encouraged to seek out the primary literature for additional information.

GE LARGE ANIMAL MODELS

The history of GE large animals originally started on near-equal footing with transgenic mice, with similar techniques across species (Gordon et al., 1980; Brinster et al., 1981). The first GE large animal species were reported in 1985 when Hammer and co-workers developed pigs, sheep, and rabbits expressing a human growth hormone (Hammer et al., 1985). This was accomplished by direct pronuclear injection of the transgene construct into a zygote. Additional transgenic livestock animals followed, expressing either economically important agricultural traits or biomedically relevant recombinant proteins in milk. However, despite these initial successes with transgenesis, the GE large animal timeline began to lag behind mice almost immediately due to differences in available reagents and resources.

One of the biggest distinctions between mice and large animal species is the availability of embryonic stem (ES) cell lines (Piedrahita, 2000). These cells, derived from the inner cell mass of blastocysts, have many positive attributes that make them ideal for gene manipulation in mice (Moreadith and Radford, 1997). First, due to self-renewal, they can be cultured for extended periods of time without significant growth rate changes or accumulation of chromosomal abnormalities. Second, ES cells are particularly amenable to efficient homologous recombination, allowing even the most challenging gene targeting events to be identified and isolated. These characteristics allow for multiple gene modifications to be easily made at the same time or sequentially. Finally, by maintaining their pluripotent nature, ES cells are able to contribute to the germline after transfer to the developing embryo.

Despite significant effort, true ES cells in large animal species have been elusive (Soto and Ross, 2016). Several reports have indicated the development of cell lines with ES

cell-like properties, but germline transmission has not yet been demonstrated in large animals generated from blastocyst injection (Kumar et al., 2015). Induced pluripotent stem (iPS) cells have also been developed for livestock species, and while there has been one report of germline transmission in chimeric pigs, the offspring failed to live (West et al., 2010, 2011). Why ES cells and iPS cells have been so challenging outside of rodent species is not clear, but possible explanations are suboptimal culture conditions (most efforts are variations on the mouse or human protocols) and differences in pluripotent states in livestock species. Even if the process could become more robust, the question remains if generating animals using these cell types would ever be worthwhile in the case of large animal species where gestation and time to sexual maturity are significantly longer than in mice. These long timeframes and the associated costs of producing and identifying chimeric animals and subsequent F1 generations would be costly. However, ES cells and iPS cells from these species may have other benefits, which will be described later. Further discussion can also be found in an excellent review by Soto and Ross (2016).

The development of mouse ES cells allowed for the rapid advance and sophistication of GE mice, while the large animal research community was left waiting for further breakthroughs before similar progress could be realized. One such event occurred with the 1997 announcement of a sheep produced by somatic cell nuclear transfer (SCNT; Wilmut et al., 1997). The SCNT process involves transfer of the nucleus from a somatic cell to an egg cell that has been stripped of its own DNA. Following fusion and activation steps, the resulting reconstructed embryos are transferred to a surrogate mother to establish pregnancy. The resulting offspring are genetically identical to the nuclear donor. This first cloned animal using SCNT, a sheep named Dolly, was an enormously important achievement, not just for the reproductive biology community, but also for those who wished to engineer the genes of non-rodent animals.

Since the SCNT process uses the nucleus of a somatic cell as a nuclear donor, any genetic modification that can be made to the somatic cell will be represented in every cell of the “cloned” animal. Because of this, most transgenic applications have moved to somatic cells and SCNT, though pronuclear injection techniques along with lentiviral infection and transposons are still used, as well (Hammer

et al., 1985; Whitelaw et al., 2004; Chou et al., 2014; Ivics et al., 2014). The ability to use selectable markers and to molecularly characterize transgene integration and copy number in the somatic cell prior to SCNT makes the process more efficient than previous methods (Schnieke et al., 1997). Despite the expectation that SCNT would open the door to more complex genetic modification in large animal species, that threshold was slowly crossed with regard to gene targeting.

Initial attempts at homologous recombination in somatic cells were impeded by several factors. First, the need for a primary somatic cell (typically, fetal fibroblasts are used) to serve as nuclear donor means that the cell culture “clock” is ticking. The longer cells are in culture, the more likely they are to senesce or develop chromosomal abnormalities and ultimately fail in the SCNT process (Lai and Prather, 2003). Second, delivery of gene targeting constructs was initially carried out using electroporation or lipid-mediated transfection. Neither method is particularly efficient for gene delivery to primary somatic cells, with only a portion of cells receiving the gene targeting construct (Ross et al., 2010). Both processes also come with significant toxicity, which in addition to affecting cell health, growth, and number, also could negatively impact SCNT. Third, even when a gene targeting construct makes it successfully into the nucleus of the cell, it is more likely to be randomly integrated than participate in homologous recombination—usually by an order of magnitude or greater (Vasquez et al., 2001). Screening for true homologous recombinants can be a costly and time-consuming task.

With these challenges, gene targeting progress was slow initially, with success limited primarily to highly expressed genes. The first gene locus to be targeted in a large animal species was alpha 1 (I) procollagen (*COL1A1*) in sheep, with the purpose of directing the specific integration of an alpha 1-antitrypsin cDNA (McCreath et al., 2000). This was soon followed by the targeted disruption of alpha-1,3-galactosyltransferase in sheep and pigs, with the hope that these animals would produce organs that could evade the hyperacute rejection response seen with xenotransplantation (Denning et al., 2001; Dai et al., 2002; Lai et al., 2002). Several years later, many of the inefficiencies of targeting genes in large animal somatic cells were overcome with the use of recombinant adeno-associated virus (rAAV) with the successful targeting of the porcine cystic fibrosis transmembrane conduc-

tance regulator (CFTR) to model human cystic fibrosis (Rogers et al., 2008b, 2008c). This is largely due to rAAV’s ability to (1) infect almost 100% of cells, (2) do so with little toxicity, and (3) deliver single-stranded targeting vectors to the nucleus (Hendrie and Russell, 2005). Even genes with low or no expression can be readily targeted, selected, and screened. Many genes have since been efficiently and precisely targeted with this approach (Hickey et al., 2011; Luo et al., 2011; Davis et al., 2014; Sieren et al., 2014; Beraldi et al., 2015; Park et al., 2015).

Another advance that helped to accelerate the pace of development of GE large animals was the completion of the various livestock species genomes (Uenishi et al., 2012; Du et al., 2014; Jiang et al., 2014). While not all of the genomes are as fully annotated as those for human or mouse, sufficient sequence data is usually available to design appropriate transgenes and gene targeting constructs. Otherwise, the orthologous human or mouse (and sometimes other mammal) genome sequences can be used to assist in obtaining gene sequences of interest.

At this point in the technology timeline, most gene additions, deletions, and point mutations were possible in somatic cells and thus could be combined with SCNT to generate many types of GE large animals. However, one potential drawback with traditional gene targeting in somatic cells is that the efficiency is only good enough to produce a single modification to a single allele. This is acceptable for cases where a heterozygous animal is preferred, such as modeling a dominant disease or producing a disease model where homozygotes are lethal or unable to breed. In cases where homozygotes are desired or multiple modifications to the same animal are needed, a long-term breeding approach to achieve these ends would be necessary, but may not be practical. More efficient methods would be needed for those applications requiring a more rapid development process.

A new era of gene modification for many species was ushered in with the use of gene editing nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 system (Kim et al., 1996; Christian et al., 2010; Jinek et al., 2012). While mechanistically different, each nuclease-based approach essentially provides the same end: a sequence-specific double-stranded DNA break (DSB). This breakage

can serve two distinct purposes. If delivered alone, the nuclease initiates a DSB, which is repaired by the error-prone nonhomologous end joining (NHEJ) process. This process results in variable sized insertions or deletions (indels), which can yield frameshift mutations, effectively knocking out the targeted gene. Alternatively, if the nuclease is provided with a homology-directed repair (HDR) template in trans, the DSB will be repaired by homologous recombination with the help of the HDR sequence, allowing for single nucleotide changes as well as specific insertions and deletions.

The use of gene editing nucleases (particularly CRISPR/Cas9) has revolutionized the development of GE mice, with their high efficiency making it possible to bypass ES cells and do the gene modification directly in zygotes (UNIT 15.7; Harms et al., 2014; Shen et al., 2013). In some cases, multiple modifications have been easily generated (Wang et al., 2013). Progress has been slower in large animal species. While each of the gene editing nucleases has been used to successfully engineer large animals, most of the work has occurred in somatic cells and paired with SCNT to produce GE offspring (Yang et al., 2011; Whitworth et al., 2014; Cui et al., 2015). Several gene disruptions (via indels) have been made by direct injection of the nuclease components in large animal zygotes; however, no HDR-mediated changes have been reported yet (Tan et al., 2016). This is not because the process will not work in large animal zygotes; rather it is more a limitation of other resources, such as sufficient zygotes, recipient animals, and facilities. These shortcomings will likely be resolved soon, and many more nuclease-edited GE large animals are sure to be seen in the near future.

ISSUES TO CONSIDER WHEN DEVELOPING A GE LARGE ANIMAL MODEL

The development of a GE large animal disease model requires significant commitment of time, resources, and facilities and thus deserves thorough planning. This section explores some of the issues to consider before beginning, including choice of species, type of modification, characterization activities, long-term production plans, and regulatory compliance.

Choice of Species

When modeling a human disease, it is important to consider which species is going

to best represent the specific human condition and also recognize any possible shortcomings. No model is ideal, so understanding the compromises before beginning is important. Wild-type livestock species have been used as surrogates for human physiology studies, and many human-like disease states have been successfully induced in these species, as well (Marshall, 1979; Steele et al., 1985; Rogers et al., 2008a; Luhken, 2012; Pinnareddy et al., 2015). The identification and use of animals with naturally occurring genetic diseases, including pigs with familial hypercholesterolemia and sheep with Gaucher and Batten diseases have served as early proofs-of-principle that large animals can be excellent models (Rapacz et al., 1986; Prescott et al., 1991; Jolly et al., 1992; Karageorgos et al., 2011). Tailored GE large animals were the next logical step.

Pigs, sheep, and goats offer advantages over rodent species due to their more human-like anatomy, physiology, metabolism, genetics, and size. While size is often cited as a disadvantage from a housing and cost perspective (large animal per diems can be 30 to 40 times those of mice), it is important to consider how important this characteristic is to fully modeling a human disease. For example, large animals are ideal for using clinical imaging equipment designed for humans. Patient-sized medical devices (stents, pacemakers, etc.) can be developed and tested, and surgical procedures can be developed, refined, and taught. Finally, large tissue samples can be collected in a longitudinal manner. None of these are possible in rodent models.

Pigs have been the predominant choice when modeling most human diseases (Rogers, 2016). Specifically, miniature pigs such as the Yucatan and Gottingen breeds have been preferred over the much larger domestic breeds (Panepinto and Phillips, 1986; Bollen and Ellegaard, 1997). Also, pigs have a significant reproductive advantage over other livestock species, with the ability to have multiple large litters per year (Table 15.9.1). Finally, as the porcine model literature continues to grow, the collective data set is becoming an important research tool in itself.

Type of Modification

Today, the types of modifications that can be engineered in large animal species are nearly unlimited. But as the world of genetic engineering explodes with new and exciting techniques, it is important to select the right modification and technique for the application.

Table 15.9.1 Reproductive Characteristics of Several Large Animal Species

Species	Gestation	Sexual maturity	Litter size	Lifespan
Minipig	114 days	6-7 months	6-10	10-15 years
Sheep	152 days	6-8 months	1-3	10-12 years
Goat	150 days	8-10 months	1-3	15-18 years

Transgenesis

Transgenesis refers to the process of adding exogenous DNA to the endogenous genome of an animal for the purpose of gaining a new function. While there are obvious transgenic applications for agriculture (enhancing growth rate), biopharmaceuticals (producing a therapeutic protein in milk), and xenotransplantation (humanizing animal organs), transgenesis has been less popular when modeling human diseases. Overexpression of dominant-negative transgenes has been useful in some cases, but the broader utility is limited (Petters et al., 1997; Jacobsen et al., 2010; Al-Mashhadi et al., 2013).

The decision of which transgenesis method to use is largely guided by whether SCNT will be employed. Pronuclear injection of zygotes is still a common practice, as is the use of lentiviruses and transposons for transgene delivery (Whitelaw et al., 2004; Chou et al., 2014; Ivics et al., 2014). Assuming zygote injection and manipulation capabilities are available, these methods are fairly straightforward. However, these methods pose several disadvantages including random transgene integration, variable copy number, potential gene silencing, and limited ability to select the desired sex. Unfortunately, these issues are nearly impossible to sort until animals have been born, making the overall process potentially very inefficient.

If SCNT is an option, the process is likely to be much more efficient. Regardless of method, cells can be screened for site of transgene integration and copy number, and the sex can be determined by the choice of donor cell line. To improve the likelihood a transgene will be expressed, site-specific integration methods can be used to target ubiquitously expressed loci, or so-called “safe harbors.” The ROSA26 locus, a popular choice for transgene location in mice, has been identified in pigs and confirmed to confer reliable expression (Li et al., 2014b). Other such loci have been considered, as well (Jakobsen et al., 2013; Ruan et al., 2015). Several methods are available to insert transgenes into a desired location. Perhaps the most efficient, though initially laborious, method is to

use gene targeting (discussed below) to introduce a recombinase-recognized acceptor sequence into a safe harbor. The resulting cell line can then be used to introduce any transgene via recombinase-mediated cassette exchange (RMCE), providing a useful long-term resource that makes up for the upfront cost and time. The Cre/lox and Flp/frt recombinases as well as the phiC31 integrase system have all been deployed in pigs and other large animal species (Bi et al., 2013; Yu et al., 2013; Li et al., 2014a). Inducible promoters have also been used in pigs (Klymiuk et al., 2012). Each of these processes requires the inclusion of a selectable marker for efficient selection. Common selectable markers in large animal species include drug resistance and fluorescent reporters, such as green fluorescent protein. Once the cell line is thoroughly characterized, it is ready for the SCNT process.

Gene targeting

Gene targeting via homologous recombination enables the ability to delete, insert, or mutate the endogenous genome in a sequence specific manner. While the process can be inefficient in large animal species, it is not the rate-limiting step it used to be, and almost any gene targeted modification that can be made in mice is now possible. Gene targeting currently requires pairing homologous recombination in somatic cells with SCNT. Key questions to be considered when designing a gene targeted large animal are: What modification is desired? How will the gene targeting construct be delivered to the cell? How will gene targeted cells be identified?

The choice of modification is dictated by the purpose of the modification. In the case of gene knockouts, gene targeting can be used to disrupt the coding region with an additional noncoding sequence, introduce a premature termination codon, delete a crucial coding sequence, or delete the entire gene in some cases. Gene targeting can also be used to insert sequences, such as selectable markers, reporter genes, or other functional elements such as human transgenes or sites for RMCE, as mentioned above. Finally, gene targeting can be

used to introduce precise point mutations of single or multiple bases.

The most commonly used methods for delivering gene targeting constructs are electroporation and viral-mediated delivery. Each has limitations that must be taken into account. For example, electroporation reaches fewer cells and can be toxic, but allows for the use of long homology arms similar to those used in traditional mouse gene targeting experiments (Ross et al., 2010). Theoretically, this should increase gene targeting efficiency and specificity. Many successful applications of electroporation incorporate promoterless positive selection markers (promoter trap); therefore, electroporation-mediated delivery is probably better suited for targeting genes that are expressed in the cell type being used (Beaton et al., 2013). The use of negative selection may or may not reduce the number of random integrants (Jin et al., 2003; Beaton et al., 2013). Alternatively, rAAV-mediated gene targeting has also been used with great success (Sun et al., 2008; Rogers et al., 2008b; Hickey et al., 2011; Luo et al., 2011). This approach reaches more cells and has little to no toxicity, but the gene targeting construct is restricted to a total of ~4.5 kilobases due to the limitation of AAV packaging. However, the shorter arms do not appear to have a detrimental impact.

Traditional gene targeting is not efficient enough to avoid the use of selectable markers, except perhaps when combined with a nuclease-mediated approach (discussed below). Depending on the specific modification and the downstream application, it may be desirable to avoid excess sequence beyond the targeted mutation. For example, the presence of a drug resistance cassette has been shown to affect the normal expression of the targeted allele (though not always), which would be undesirable in the case of a point mutation (Pham et al., 1996). This can be addressed, just as in mice, by planning for recombinase- or transposon-mediated excision in the original targeting construct design, thereby leaving little or no residual “footprint” (Kaartinen and Nagy, 2001; Carlson et al., 2011). However, in mice, excision would typically be accomplished by crossing the newly made animal with an existing mouse line that expresses a recombinase driven by a ubiquitous, or perhaps a tissue-specific, promoter. In large animals, it probably makes more sense to excise or remove the unwanted sequence before SCNT rather than go through the costly and laborious process of generating and, more importantly, maintaining recombinase-

expressing animals. Except in specific cases where breeding frequency, facilities, and budgets allow it, recombinase-expressing large animals may not be practical.

Gene editing

Nuclease-mediated gene editing has been an exciting addition to the large animal genome modification toolbox in recent years. ZFNs and TALENs have been used to develop GE livestock for a range of applications, including agriculture, xenotransplantation, and biomedical modeling (Tan et al., 2016). Because of the complexities of design and execution involved with the use of these two chimeric nucleases, most researchers are likely to prefer the ease-of-use of the more recent CRISPR/Cas9 system. Therefore, this overview will only discuss the latter system; however, ZFN and TALEN applications in large animal species have been thoroughly covered in reviews (Carlson et al., 2013; Tan et al., 2016).

CRISPR/Cas9 is derived from an endogenous anti-viral system from *Streptococcus pyogenes* and has been engineered to consist of two components (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013). The first is a guide RNA of ~20 nucleotides that is designed to hybridize with the target of choice. The only requirement for the guide RNA is that the targeted sequence be proximal to a protospacer adjacent motif, which in the case of the *S. pyogenes* Cas9 is 5'-NGG-3'. The second component is the Cas9 nuclease itself, which forms a complex with the guide RNA to target a specific DNA sequence which is then cleaved. These are the only components necessary if a simple gene knockout is the goal. For specific insertions, deletions, and point mutations, an additional HDR template is needed. HDR template design can be very simple, with short, asymmetric homology arms; however, conventional gene targeting constructs pair well with CRISPR/Cas9 for some applications, too (Richardson et al., 2016). The efficiency of the CRISPR/Cas9 system depends on the sequence context and delivery approach but is typically high enough to achieve some measure of bi-allelic modification. If working in cells, the high efficiency may eliminate the need for a selectable marker, though if the downstream application will tolerate the presence of one, it will make selection and animal production more efficient. Otherwise, excess animals will need to be produced via SCNT in order to find the desired genotype. For most biomedical modeling applications, avoiding a

selectable marker may not be worth the extra time and expense of that screen.

Perhaps the most exciting opportunity CRISPR/Cas9 provides is the ability to perform gene modification directly in zygotes, eliminating the need for SCNT and speeding up the process, at least in theory (Redel and Prather, 2015). Despite the increased efficiency of genome modification, the production of numerous animals will be required, which then must be molecularly characterized to identify the resulting mutation. However, this potential trade-off of increased genome modification for decreased efficiency on the animal production side may be acceptable for researchers with appropriate funds and facilities. Also, unlike similar work in mice, large animal zygotes are not as easily sourced. Naturally produced embryos require regular access to a large number of breeding animals and well-managed embryo collection logistics. This is particularly challenging if working in specialized breeds, such as miniature pigs. In vitro fertilized embryos are more easily obtained but are challenging in some large animal species. For example, in vitro fertilized pig embryos suffer from a high level of polyspermy, which must be avoided for the process to be efficient (Wang et al., 1998). Oocyte availability is typically limited to the agriculturally important breeds, which for pigs is the potentially undesirable larger breeds. Many of these shortcomings may become more manageable in the future, but currently, it is important to carefully choose methods that are practical for one's budgetary and infrastructure realities.

GE Animal Characterization

A significant amount of space in this overview (and the literature, as a whole) has been spent discussing the various techniques developed to speed up the process of developing GE large animals, but this is a bit shortsighted. Developing the GE large animal is not (and has not been for some time) the rate-limiting step or the most challenging part of the process. Characterization of the resulting animal will consume the vast majority of the total time and resources required for the project.

Long before an animal is even born, it is important that an interdisciplinary team be in place to guide the characterization activities. In addition to molecular biologists and reproductive scientists, individuals with human clinical expertise and veterinary experience must consider how the human disease will present in a large animal species, how the animal will be medically treated when the phenotype mani-

fest, and what the appropriate end points will be with regard to animal welfare. Once the animal is born, it is too late for these discussions.

Molecular and functional characterizations are typically the first steps. Do the animals have the expected or intended modification? This is not always a given. For example, random integrants can evade selection screens at the cell culture level and end up as nuclear donors in SCNT. Careful assessment using PCR, Southern blots, and DNA sequencing technology are necessary. Once the intended sequence is confirmed, does the mutant gene produce the intended functional outcome? Some targets have obvious biochemical or enzymatic readouts, but others are more challenging to confirm. Furthermore, knockout modifications may result in no full-length protein product being produced, but alternatively-spliced variants could still be generated and have unintended consequences. Gene knockouts that rely on premature termination codons, such as NHEJ-mediated indels and gene disruption strategies, often trigger nonsense-mediated altered splicing, yielding unexpected mRNAs and truncated proteins (Wang et al., 2002). Finally, a close look at tissue samples collected from biopsy or necropsy can often be the first confirmation that disease phenotype is present; thus, utilizing the expertise of comparative pathologists is highly recommended.

Imaging technologies can be an important part of characterizing a large animal model. For example, computed tomography (CT) and magnetic resonance imaging were key for detecting the presence of tumors in a mutant TP53 porcine cancer model (Sieren et al., 2014). In addition to helping with a diagnosis, the imaging data guided the postmortem collection of tissues, most of which would have been missed on traditional necropsy. Another example is the use of positron emission tomography and CT to characterize a porcine model of cardiovascular disease (Al-Mashhadi et al., 2013). In both models, the imaging technology can be used for monitoring the initiation and progression of disease, as well as the possible reversal of disease when testing treatment modalities. Having access to imaging resources that have been developed for humans significantly increases the value and utility of the animal as a translational model.

Behavioral impairment is a feature of many neurological diseases, so the ability to assay cognitive and motor skills in large animals will be important. While the use of such tests in mice is well established and robust, their

application to large animal species is limited; however, progress is being made. Learning and memory in normal pigs have been tested in T-mazes and spatial hole-board discrimination tasks (Elmore et al., 2012; Gieling et al., 2012), and these tests are now being deployed in several GE models (data not published). A cognitive testing system has been developed to assess a transgenic sheep model of Huntington's disease (McBride et al., 2015). Gait analysis was used to characterize the development of phenotypic abnormalities in a porcine model of ataxia-telangiectasia (Beraldi et al., 2015). As more GE large animal models are developed, additional behavioral assays will certainly be developed or scaled from existing rodent versions. It is important to note that large animal behavioral apparatuses require significant space, so appropriate facilities and planning are required.

Animal Production Method

Another important consideration is the method of larger scale animal production. In other words, will a breeding herd be established and maintained or will subsequent animals be produced by SCNT? Both options have advantages and limitations.

Creating a breeding herd requires appropriate animal facilities and trained husbandry staff. Most research facilities are not capable of maintaining breeding herds, though some agriculture-based universities and contract research organizations, as well as large animal modeling companies, are well qualified for these pursuits. Another factor to consider is access to breeding stock in sufficient numbers and genetic diversity. Scheduling the delivery of specific animals is challenging due to the typically longer gestation times found in large animals species and is compounded by accommodating estrous cycles. Therefore, in order to be able to provide adequate animal numbers on a research-friendly schedule, it helps to have a large number of potential breeders from which to choose. It is also important that the breeding animals have appropriate genetic diversity in order to avoid issues of inbreeding, which are common in livestock species. A final consideration is the impact of the disease on breeding. In the case of autosomal recessive diseases, heterozygotes can be maintained and bred. While only 25% of offspring will be homozygotes, such crosses typically provide wild-type animals as controls and heterozygotes to use as future breeders. For diseases in which phenotype severity and age of

onset allow, homozygotes (or heterozygotes, if autosomal dominant) may also be used as breeders.

In addition to generating the initial founder animals, SCNT offers some advantages as a larger scale production method. In the example of a severe autosomal dominant disease, cloning the animal may be the only way. Also, in cases of animals with multiple modifications on different chromosomes, production by SCNT would be more efficient than breeding. Producing animals by SCNT requires a smaller herd (recipient animals are still required) and can be scheduled more predictably. Furthermore, there are some cases where it may be advantageous to study genetically identical animals. If appropriate baseline genetic cell lines are maintained, it may also be possible to generate genetically identical wild-type control animals, with the only difference being the modification. Conversely, these attributes can be seen as a lack of genetic diversity and lack of littermate controls, which may be unwanted for some experiments. Perhaps the biggest drawback of SCNT is the occasional production of defective animals, which is believed to be the result of incomplete nuclear reprogramming during the SCNT process (Zhao et al., 2010). These present differently across species but have the potential to negatively impact animal production and phenotype interpretation (Schmidt et al., 2015). If ES cells or iPS cells can ever be fully developed in large animal species, perhaps they would provide a more easily reprogrammed cell to serve as a nuclear donor (Soto and Ross, 2016). Ultimately, each disease model is different, but when circumstances allow, production by breeding is typically preferred.

Regulatory Compliance

The final consideration for working with GE large animals is regulatory compliance. Requirements may differ among countries, but this section focuses on the United States. Only experiments that have been approved by an Institutional Animal Care and Use Committee should be conducted. It is also important that researchers follow the Animal Welfare Act and the guidelines presented by the US Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) to protect animal welfare. Readers are encouraged to view their website, which is an excellent resource (<https://www.aphis.usda.gov>). Furthermore, the National Research Council publishes the Guide for the Care and Use

of Laboratory Animals, which provides a framework for animal housing, husbandry, and management (National Research Council, 2011). Finally, because the US Food and Drug Administration (FDA) regulates GE animals, researchers must comply with their Guidance for Industry #187: Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs (FDA, 2009).

THE POTENTIAL IMPACT OF A GE LARGE ANIMAL MODEL

The first GE large animal model of a human disease was a transgenic porcine model of retinitis pigmentosa, which was reported in 1997. Numerous animal models have been developed since then, but rather than provide the entire history here, readers are encouraged to seek out several recent reviews for a comprehensive list (Flisikowska et al., 2014; Rogers, 2016). Instead, this section is used to highlight an example of the significant impact a large animal model has had on one disease: cystic fibrosis (CF).

CF is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Welsh et al., 2001). The *CFTR* protein is responsible for transporting chloride and bicarbonate across epithelia in various tissues. Well over 1000 CF-associated mutations have been identified in *CFTR*, but the most common is the deletion of Phe508 (F508del), which causes *CFTR* to be misprocessed and degraded rather than being properly delivered to the surface of the epithelial membrane. CF affects multiple organs including the pancreas, intestines, and sweat glands; however, it is infection and inflammation in the lungs that are responsible for most of the morbidity and mortality. With time, bacteria permanently colonize the airways, and patients develop persistent respiratory symptoms and progressive airway inflammation; most patients die of respiratory failure.

Several CF mouse strains carrying null and missense mutations were developed (Grubb and Boucher, 1999); however, CF mice fail to develop the lung or pancreatic disease seen in patients. Because of the many advantages that have already been described above, a CF pig was developed. Combining gene targeting and SCNT, two different mutations were introduced to the endogenous *CFTR* gene: a null mutation and the common F508del (Rogers et al., 2008b). Initial characterization demonstrated that CF pigs develop all of the

key clinical manifestations seen in patients, including lung disease.

The CF pig model has since been used to address several fundamental questions about the innate immune system and disease pathogenesis (Stoltz et al., 2015). For example, there has been a long-standing paradox of whether the inflammation in the lung establishes an ideal environment for subsequent bacterial colonization or if it is the presence of bacteria that leads to inflammation. Studies in newborn piglets demonstrated that CF animals have a host-defense defect, allowing early bacterial infection in the absence of inflammation (Stoltz et al., 2010). The porcine model was used to demonstrate this defect is caused, in part, by a reduction in the pH of airway surface liquid (ASL), resulting in the inhibition of antimicrobial peptides present there (Pezzulo et al., 2012). In another study, the mucus of CF pigs was found to not detach from submucosal glands in the airway, preventing normal clearance of pathogens from the lung (Hoegger et al., 2014). These and other studies have yielded important insights into the initiating events of the CF lung disease and provided clues for potential therapeutic intervention. Importantly, none of these experiments would have been possible with murine models.

CONCLUSIONS

GE large animal models are clearly gaining in popularity, and for good reason. As presented above, they offer many advantages. However, realizing those advantages requires a significant commitment of time, effort, and resources. Much work remains to bring the research infrastructure for large animal species on par with human and mouse resources. Many of the postgenomic tools have lagged for large animals; however, efforts are underway to remedy that (Schook et al., 2015). Similarly, research reagents such as antibodies and biomarkers are not as readily available, but many labs and companies are beginning to fill these voids. Finally, while the lack of ES cells and iPS cells no longer hinders the development of new animals, it does pose a problem for testing various cell therapies.

Large animal models will also need to gain acceptance. In a world dominated by GE mouse models and human cell systems, shifting the paradigm will be difficult. It is important to note that the development of large animal models should not be interpreted as a push to replace existing models. In fact,

having more models of disease allows for comparative biology approaches that can reveal findings that studying a single model cannot. CF is again an excellent example. As described previously, the lungs of CF pigs have a host defense defect that results in lower pH of the ASL. This is not the case in CF mice, which also do not develop lung disease. Exploring these differences led to the identification of a proton pump that contributes to the acidic ASL in CF pigs, but when expressed at low levels (as it is in CF mice) prevents this acidification (Shah et al., 2016). In addition to explaining why the CF mice do not develop lung disease, it also revealed this proton pump as a possible therapeutic target. This will not be the last example of a multispecies comparison providing key discoveries, and stories like these will hopefully help large animal species gain acceptance alongside their smaller counterparts.

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